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A MicroBio Reactor for Hydrogen Production

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A MicroBio Reactor for Hydrogen Production

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Abstract

The purpose of this work was to explore the potential of developing a microfluidic reactor capable of enzymatically converting glucose and other carbohydrates to hydrogen. This aggressive project was motivated by work in enzymatic hydrogen production done by Woodward *et al.* at ORNL. The work reported here demonstrated that hydrogen could be produced from the enzymatic oxidation of glucose. Attempts at immobilizing the enzymes resulted in reduced hydrogen production rates, probably due to buffer compatibility issues. A novel in-line sensor was also developed to monitor hydrogen production in real time at levels below 1 ppm. Finally, a theoretical design for the microfluidic reactor was developed but never produced due to the low production rates of hydrogen from the immobilized enzymes. However, this work demonstrated the potential of mimicking biological systems to create energy on the microscale.

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Introduction

A major limitation of microdevices, particularly those used in remote sensing applications, is the limited lifetimes and poor power-to-weight ratio of the batteries that generally serve as the power source. Miniaturized fuel cells, which can provide power as long as fuel is present and generally have better power densities than batteries, are being developed to address this shortcoming. However, fuel availability is a major issue in the development of these miniature fuel cells. Current conceptual designs call for the incorporation of a “fuel tank” of hydrogen or methanol to feed the fuel cell, thus limiting the duration of power generation. If, however, the fuel passed to the fuel cell could be harvested from the environment in which the microdevice resides, power could be generated indefinitely.

Living organisms have successfully been harvesting and utilizing energy from the environment for billions of years. The purpose of this work was to explore the possibility of mimicking biological systems in order to harvest energy from the environment to power microsystems. Specifically, the vision of this work is to eventually develop an enzyme-based microfluidic reactor that can convert environmentally available carbohydrates to molecular hydrogen. This aggressive one year project was funded out of the Science and Technology Directorate at Sandia National Laboratories and was intended to be a proof-of-principle study. The work presented here demonstrates that hydrogen can be produced from glucose using enzymes in solution. However, many challenges still remain in order to develop a functional microbioreactor for hydrogen production.

Though this project was motivated by the possibility of developing a novel “ever-lasting” power source for microdevices, biologically converting carbohydrates to hydrogen could play a role in the development of the “Hydrogen Economy.” To fully reap the benefits of a hydrogen-based economy, the hydrogen must be generated from renewable resources and in a manner that does not result a net emission of carbon dioxide. The biological conversion of environmental stores of carbohydrates to hydrogen fits these constraints. Thus, an important side benefit of this work is that it could potentially be applicable to the development of a distributed hydrogen generating infrastructure

Stores of carbohydrates, commonly present as polymers of glucose (e.g., starch and glycogen) or polysaccharides (e.g., sucrose and lactose), are widespread in the environment and biologic systems. Therefore, cells have evolved remarkable metabolic machinery that enables them to generate energy from the oxidation of glucose (and other organic molecules). Thus, in order to mimic these biological systems and harvest energy from environmental glucose, it is important to understand the specifics of how cells utilize glucose as an energy source.

Cells, of course, do not harvest energy from glucose by generating hydrogen. Instead, they gain energy by coupling the oxidation of glucose to the generation of ATP (the “energy currency” of the cell). Some ATP is directly generated from glucose oxidation by substrate level phosphorylation. However, the majority of ATP is generated indirectly

through a process known as oxidative phosphorylation. In this process, electrons that are removed during the oxidation of glucose are first transferred to the oxidized form of the reducing equivalent NAD^+ (or NADP) to create NADH (or NADPH). These reduced “electron carriers” then transfer their electrons to the electron transport chain (a series of electron-transferring proteins), which eventually results in the generation of ATP and the reduction of molecular oxygen to water

Glucose is primarily metabolized through three central metabolic pathways: the glycolytic pathway, the pentose phosphate cycle and the tricarboxylic acid (TCA) cycle. When glucose is transported into the cell, it is phosphorylated to glucose-6-phosphate. Based on the needs of the cell, a “decision” is made to either send glucose-6-phosphate through the pentose phosphate cycle or continue down the glycolytic pathway. The pentose phosphate pathway is used to generate NADPH as well as produce pentose sugars that serve to create the ribose-sugar backbone of nucleic acids (RNA and DNA). Under normal conditions, most cells will route approximately 30% of the imported glucose through the pentose phosphate cycle. The remaining glucose-6-phosphate is passed through the glycolytic pathway, which terminates with the production of pyruvate. The degradation of glucose-6-phosphate to pyruvate results in the net generation of 2 moles of ATP and 2 moles of NADH per mole of glucose. If there is a sufficient amount of molecular oxygen around, and if the cell requires additional energy, the pyruvate resulting from glycolysis can be passed into the TCA cycle to generate more NADH and thus ATP. If molecular oxygen is not present or is present in low concentrations, the cells are unable to regenerate NAD^+ (or NADP) from the electron transport chain. Under these circumstances, NAD^+ and NADP are regenerated by reducing pyruvate to lactate (or ethanol, in the case of yeast).

Unfortunately, despite the remarkable ability of cells to harness energy from glucose, it is currently impossible to integrate this energy generation with human-developed technology. Thus, the challenge is to use the biologically reaction pathways as a model for creating a useable fuel source. Fortunately, some microorganisms have evolved the ability to metabolically generate molecular hydrogen. The reasons that these organisms produce hydrogen aren’t particularly well understood. However, metabolically-produced hydrogen occurs under strictly anaerobic conditions and thus it is thought that the generation of molecular hydrogen is a way for cells to regenerate NAD^+ (and NADP) from NADH (and NADPH) akin to the way the oxidized electron carriers are regenerated in other situations by the reduction of pyruvate to lactate. A novel hydrogenase enzyme encoded by the thermophilic bacterium *Pyrococcus furiosus* has been isolated and found to catalyze the production of molecular hydrogen using NADPH as the electron donor (see Figure 1). The work reported herein used this enzyme as part of multi-enzyme system to generate hydrogen from glucose. Unfortunately, since hydrogen production evolved under anaerobic conditions, the hydrogenase enzyme is extremely sensitive to oxygen, which makes working with it challenging.

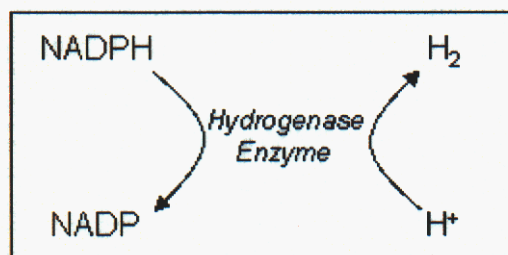


Figure 1: The biological production of hydrogen catalyzed by a hydrogenase from *Pyrococcus furiosus*.

The underlying concept of this work is to mimic biological systems by coupling glucose oxidation to the reduction of NADP to NADPH. However, instead of using NADPH to generate ATP (as would generally be done in cellular systems), NADPH will serve as an electron donor for the hydrogenase enzyme in order to produce molecular hydrogen (see Figure 2)

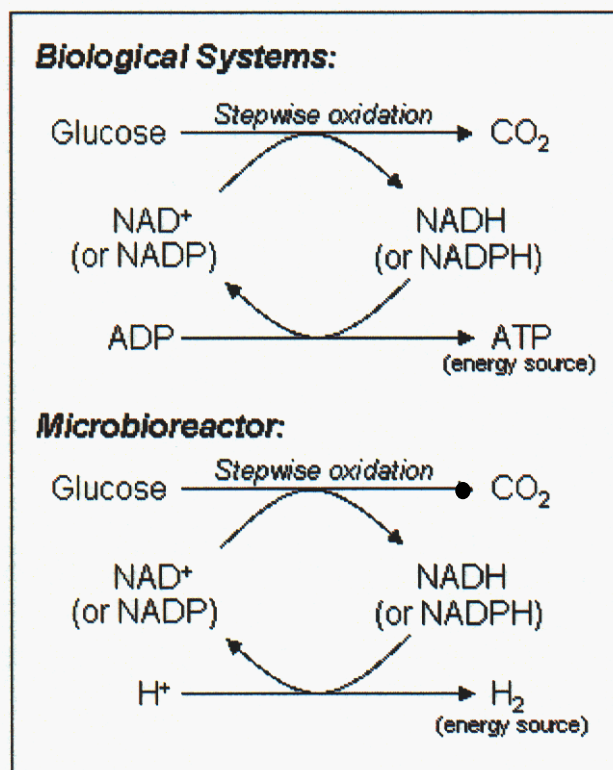


Figure 2: Mimicking biological systems to produce hydrogen.

Work in this area is not new. A group at Oak Ridge National Laboratories (ORNL) has previously used the enzymes of the PPC to convert glucose-6-phosphate to hydrogen [3]. The first two enzymes in the PPC (see Figure 3) comprise the oxidative branch of the PPC and are responsible for the two-step conversion of glucose-6-phosphate to ribulose-5-phosphate. Each of these enzymatic steps results in the evolution of one mole of NADPH per mole of glucose-6-phosphate. Enzymes of the non-oxidative branch of the PPC convert

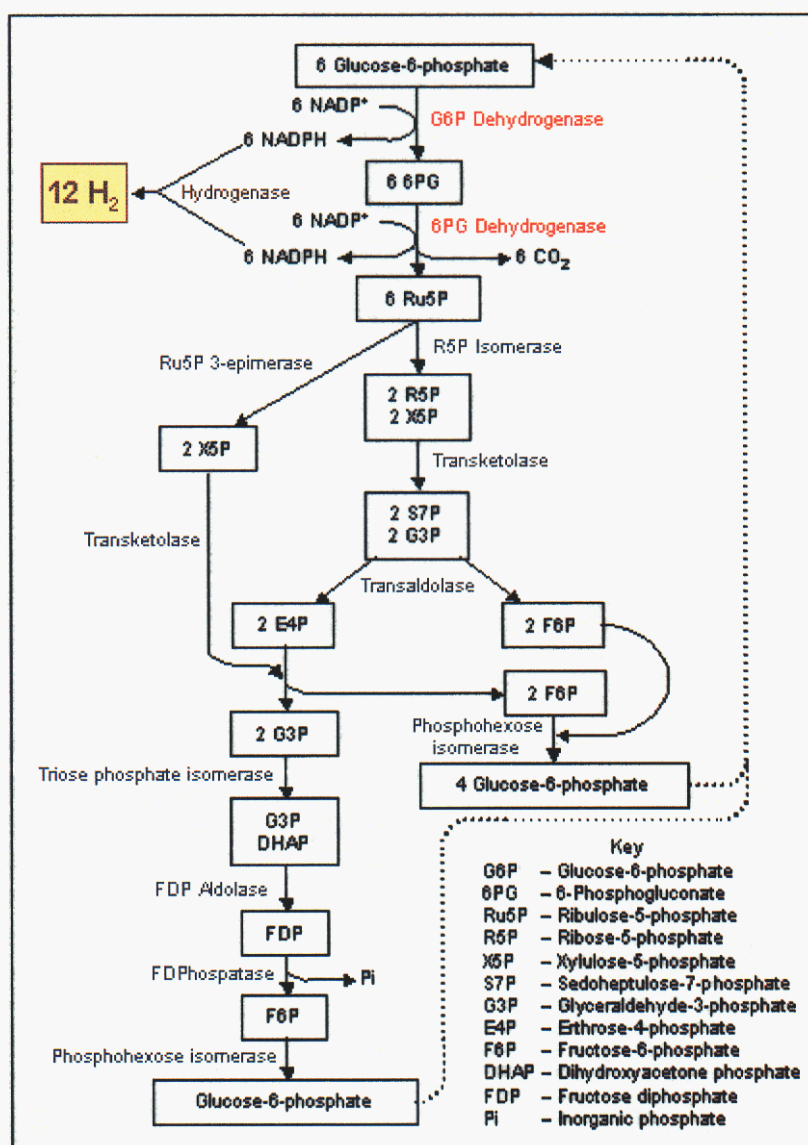


Figure 3: Enzymatic pathway for the production of H_2 from glucose-6-phosphate. The enzymes listed in red are the enzymes of the oxidative branch of the Pentose Phosphate Pathway (PPC); those in blue are from the non-oxidative branch of the PPC. The enzyme catalyzing H_2 production from NADPH is shown in purple. Figure adapted from Heyer N, Woodward, J. 2001. Efficient hydrogen production using enzymes of the pentose phosphate cycle. Proceedings of the 2001 DOE Hydrogen Program Review.

ribulose-5-phosphate to a variety of vital pentose phosphate sugars that are used in the synthesis of DNA, RNA and aromatic amino acids. Additional reducing equivalents can be produced by recycling the pentose phosphate sugars back through the pathway [1,2]. Remarkably, due to the cyclic nature of the pathway, all of the electrons within the carbohydrate can be used to generate the reducing equivalent NADPH and thus are accessible for hydrogen production. By combining the enzymes of the oxidative branch of the PPC with the hydrogenase in a 2 ml batch reactor, two moles of H_2 per mole of glucose-6-phosphate were produced, a 17% stoichiometric yield (the maximum theoretical yield of molecular hydrogen from glucose is 12 moles of hydrogen per mole of glucose). When the

enzymes from the non-oxidative branch of the PPC were added to the batch reactor, 11.6 moles of H₂ per mole of glucose-6-phosphate were produced, a 97% stoichiometric yield. The maximum rate of hydrogen production was 425 nmol/hour, the equivalent of a theoretical 35 μ W of power output.

The work performed at ORNL was revolutionary but has limited applicability due to the batch nature of the hydrogen generating reactor and the low rate of hydrogen generation. The work performed in this project sought to expand on the work at ORNL by developing a microreactor where the key enzymes would be immobilized in microfluidic channels. The vision of this project was to take the first steps towards the creation of a system that could produce hydrogen continuously (as opposed to in batch form) and at higher rates than observed in the ORNL experiments (due to high enzyme loading of the immobilized enzymes and, eventually, enzyme engineering).

No work in this area had been attempted at Sandia prior to the initiation of this project. Despite the short 12-month timeframe and the limited budget, this project did achieve some successes that could merit looking at the technology in more detail in the future. These successes included:

1. An in-line hydrogen detection system was adapted from a plant physiology analysis system.
2. The acquisition of all of the necessary biological components of the system.
3. The hydrogenase enzyme was used to produce hydrogen in solution in the absence of other enzymes using both artificial and natural (NADPH) electron donors.
4. Hydrogen was produced from glucose where all reaction components were in solution.
5. Enzymes were immobilized to silica beads in order to develop and immobilization protocol. Immobilization resulted in a 20-fold decrease in activity. Attempts to increase activity of the immobilized enzymes were not successful.
6. A conceptual design for the microreactor was developed.

Results and Discussion

One of the most challenging aspects of this project was the requirement to rapidly and inexpensively develop an in-line hydrogen detection system that would enable the quantification of the hydrogen produced in the experiments. Unfortunately, the majority of the systems that have been used by other researchers rely on expensive (more than \$20,000) micro-gas chromatograph systems. As this was well out of the project's budget, an analysis system was obtained from Qubit Systems (www.qubitsystems.com) that is designed for high school biology labs to study nitrogen fixation in plants. This detection system was adapted to this project and was able to quantify the production of hydrogen from our reaction vessel (see Figure 4). The detector itself depended on being saturated with oxygen in order to function correctly. Thus, oxygen was plumbed into the detection system downstream of

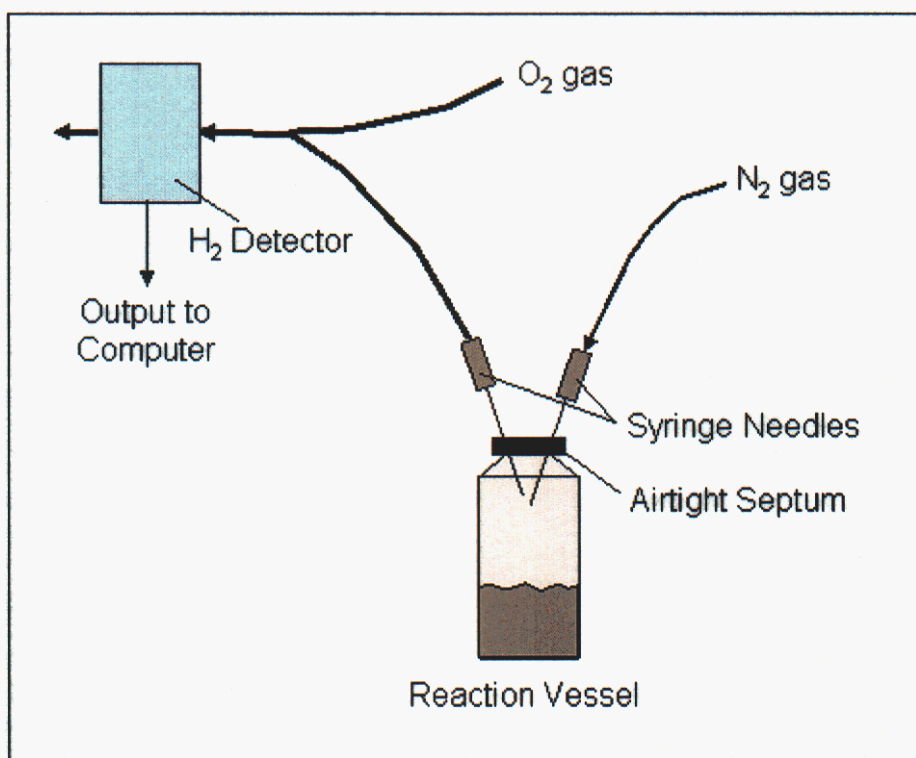


Figure 4: Schematic of the experimental system.

our reaction vessel (recall, the hydrogenase enzyme is extremely sensitive to oxygen). The detector was able to detect molecular hydrogen in the range of 0 – 2000 ppm. The detector was calibrated using pure hydrogen gas standards and the response of the detector to hydrogen was found to be non-linear. Data management software provided with the detector enabled the quantification of hydrogen concentrations by comparing the output signal to a standard curve.

Most of the enzymes, substrates and electron donors used in this project were obtained from commercial providers. However, the hydrogenase enzyme, the key to making this system work, is not available commercially. Fortunately, one of the key scientists working in the field, Mike Adams at the University of Georgia, agreed to furnish the enzyme. Unfortunately, the enzyme was very costly. Thus, if this reactor system is to be developed in the future, it will be desirably to find a less expensive way of obtaining the enzyme.

As mentioned previously, the hydrogenase enzyme is extremely sensitive to oxygen which made using the enzyme in experiments challenging. The frozen hydrogenase was transferred to a nitrogen glove box, thawed, and the appropriate volume was transferred to a 5 ml serum bottle containing reaction buffer (sodium phosphate). The bottle was capped with an air-tight septum and removed from the glove box. In order to initiate the hydrogen production reaction, the remaining reaction components were added to the serum bottle through the septum using a syringe. For all experiments run in this project, a total reaction volume of 2 ml was used. This small reaction volume not only minimized the use of the

high-cost reaction components, but made the results more applicable to the intended future application of powering a microdevice.

In order to test our experimental system and confirm the ability of the hydrogenase enzyme to produce hydrogen, a series of experiments were conducted using the artificial electron donor methyl viologen (see Figure 5). The reaction mixture contained two units of the hydrogenase enzyme (one unit is defined as the amount of enzyme required to generate one micromole of hydrogen per minute at 80°C) in 2 ml of sodium phosphate buffer with 1 mM of methyl viologen. The same experiment was repeated several times using the native electron donor NADPH at 1 μ M (NADPH is very expensive and thus was used at low concentrations, when possible). Results were nearly identical for both systems; a maximum production rate of 500 nmole of hydrogen per hour was obtained with yields (in the case of experiments with NADPH) exceeding 90% (data not shown).

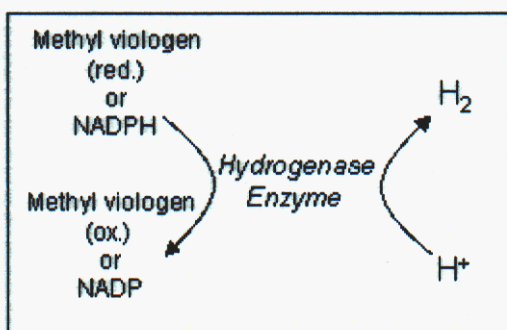


Figure 5: Hydrogen production from NADPH or methyl viologen.

The revolutionary work done by Woodward *et al.* at ORNL used all of the enzymes (a total of 10) from the Pentose Phosphate Cycle along with the hydrogenase enzyme in order to obtain a 97% stoichiometric yield of molecular hydrogen from glucose-6-phosphate. In the development of our microreactor, we wanted to minimize the number of enzymes in our reaction vessel so as to limit the number of components that would have to be optimized when we attempted to immobilize the enzymes. In addition, Woodward used glucose-6-phosphate in order to generate hydrogen whereas we preferred to use non-phosphorylated glucose. Thus, in this work, rather than using the enzymes of the Pentose Phosphate Cycle, we used the enzyme glucose dehydrogenase. Glucose dehydrogenase catalyzes the oxidation of glucose to gluconic acid with the concomitant reduction of NADP⁺ to NADPH. Therefore, by combining glucose dehydrogenase with the hydrogenase enzyme, glucose can be converted to hydrogen (see Figure 6). The disadvantage of our system compared to that of ORNL is that the oxidation of glucose to gluconic acid results in the removal of only two electrons from glucose. Thus, the maximum stoichiometric yield we could obtain in this system is just 8% (1 mole of molecular hydrogen per mole of glucose). Of course, to truly realize the vision of this project, all of the enzymes used by Woodward *et al.* will eventually need to be incorporated into our microreactor. In addition, the enzymatic system required to convert glucose to glucose-6-phosphate will need to be incorporated into the finished reactor. This

required phosphorylation step will likely be a major challenge in developing an autonomous, continuously operating microbioreactor for hydrogen production.

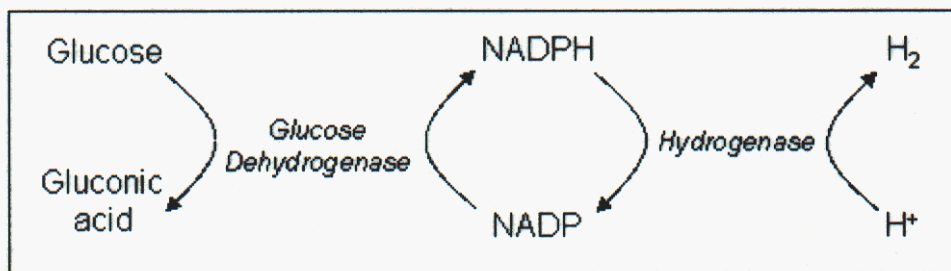


Figure 6: Enzymatically catalyzed hydrogen production from glucose.

A series of experiments were run in order to demonstrate that hydrogen could be produced from glucose. A 2 ml reaction volume was used for all experiments. The reaction mixture was made-up with ~ 30 units of hydrogenase, ~ 10 units of glucose dehydrogenase and 1 micromole of NADPH in sodium phosphate buffer at pH 7.5. The reaction was initiated by the addition of 10 micromoles of glucose and was carried out at a variety of conditions. The maximum production rate of hydrogen was found to occur at 50°C. Hydrogen production was immediately observed after glucose addition and continued for nearly four hours. After four hours, hydrogen production stopped, presumably indicating the depletion of the glucose. After the hydrogen production had been stopped for two hours, an additional 10 micromoles of glucose was added to the reaction chamber and hydrogen production was again observed. The maximum rate of hydrogen production peaked at ~ 500 nmole/hour and a stoichiometric yield of ~ 8% was observed, the maximum yield based on the conditions that the experiments were run under (see Figure 7).

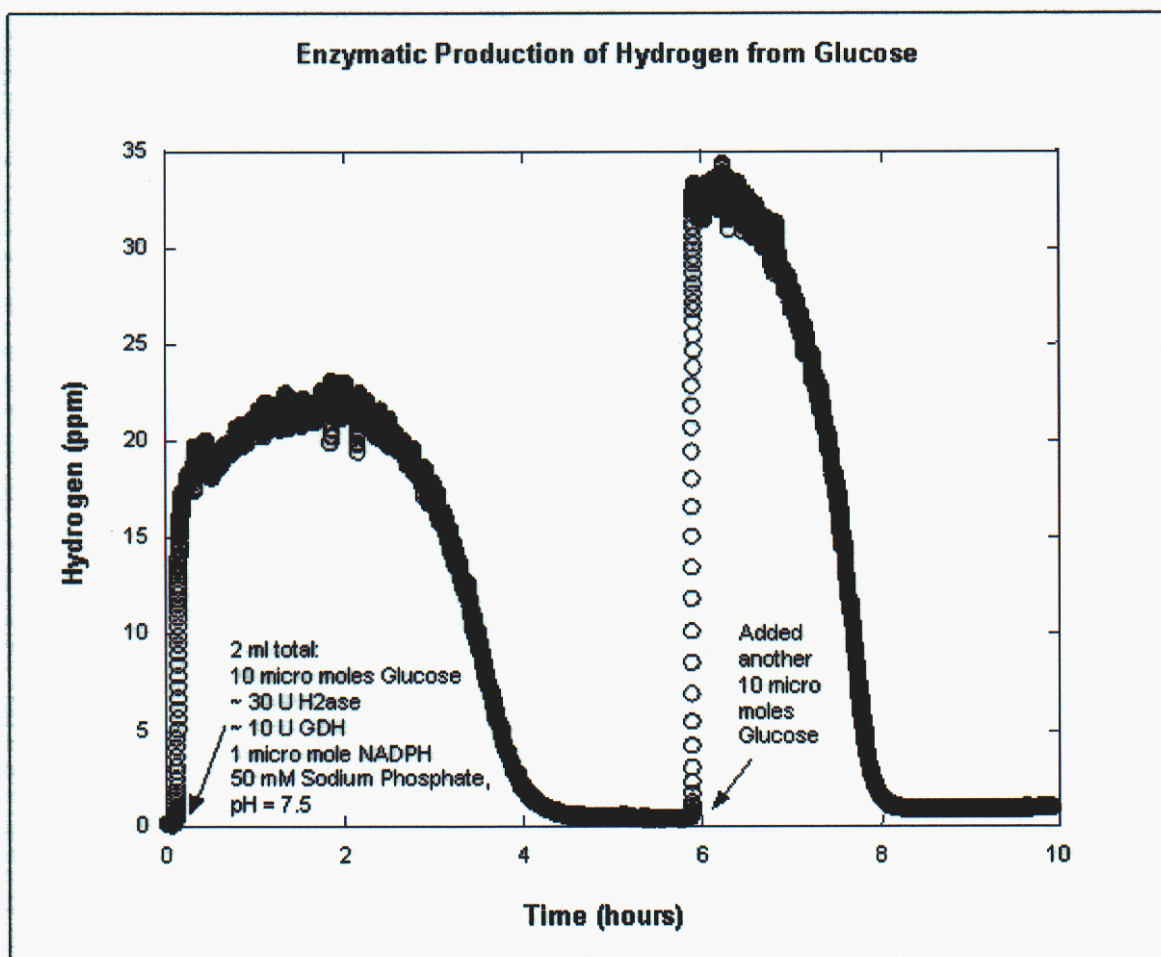


Figure 7: Hydrogen production from glucose using enzymes in solution.

In order to realize the vision of this project the enzymes that were used in solution to generate hydrogen will have to be immobilized into a microreactor. Thus, numerous experiments were conducted to try and develop a protocol to immobilize the enzymes onto porous silica beads. The hydrogenase and glucose dehydrogenase enzymes were immobilized onto 7 μm diameter, 1000 Angstrom porous silica beads with an aminopropyltriethoxysilane-glutaraldehyde cross-linking step. The silica beads loaded with the immobilized enzymes were added to 800 microliters of buffer containing 10 micromoles of glucose. One micromole of NADPH was added to the reaction mixture to initiate the reaction and the hydrogen production was monitored. Hydrogen evolution was observed but at a rate roughly 20 times less than that observed when enzymes were used in solution. After hydrogen production ceased, an additional 10 micromoles of glucose was added to the reaction mixture. However, no additional hydrogen production was observed. Clearly, and not unexpectedly, the immobilization of the enzymes had a major impact on the activity of the enzymes (see Figure 8).

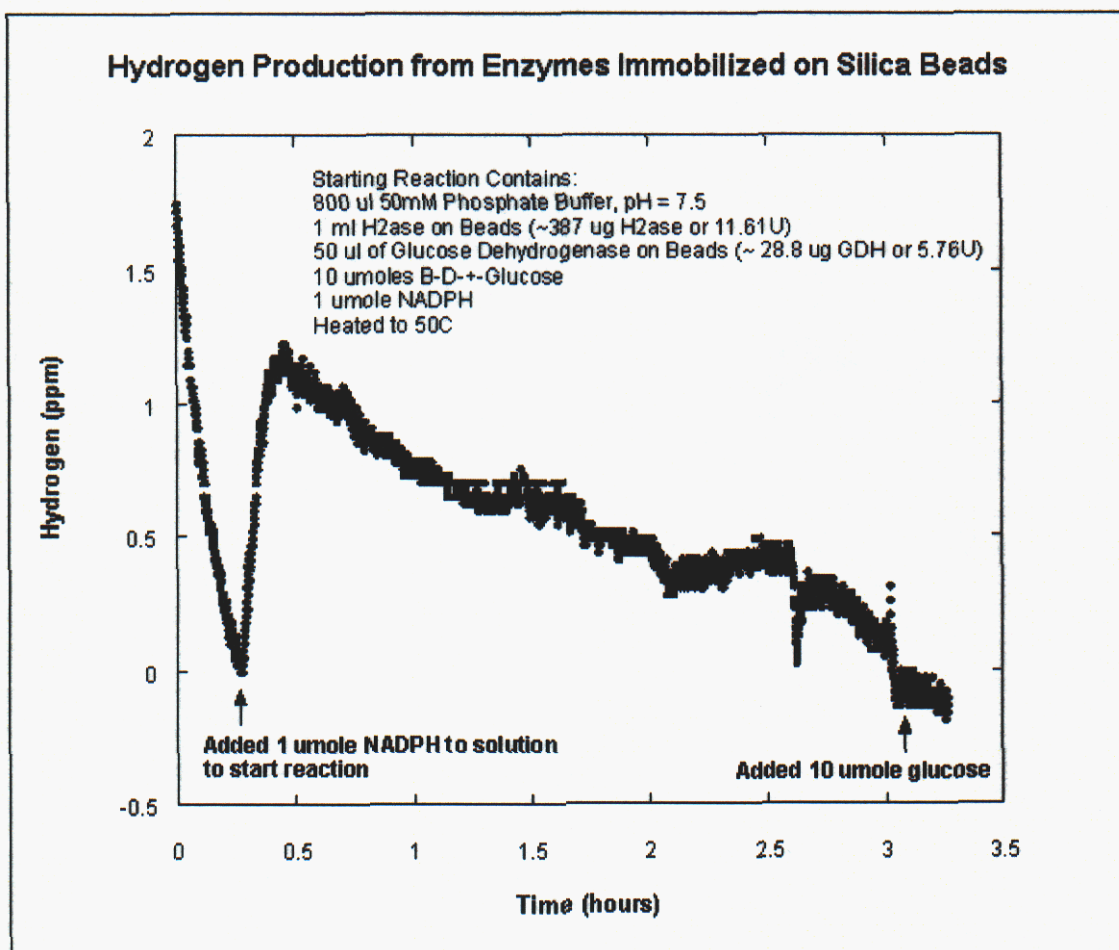


Figure 8: Hydrogen production from glucose using immobilized enzymes.

It was thought that a major reason for observed decrease in activity of the immobilized enzymes was due to the fact that the hydrogenase enzyme was supplied in a Tris buffer. The amine groups in the Tris buffer probably interfered with the cross-linking procedure. Thus, the enzyme was moved from a Tris buffer to a PBS buffer using a buffer exchange column. Glucose dehydrogenase was also suspended in a PBS buffer and the immobilization procedure was done again. This time, however, the system did not produce any hydrogen. Thus, hydrogenase and glucose dehydrogenase (in soluble, non-immobilized form) were added back to separate reaction vessels containing the immobilized enzymes to determine if which (if either) of the immobilized enzymes were functional. This experiment demonstrated that the immobilized glucose dehydrogenase was still active whereas the immobilized hydrogenase had no activity. Thus, it is clear that more work needs to focus on the immobilization of hydrogenase. Unfortunately, time ran out on this project before this area could be explored further.

As this project was unable to achieve high yields of hydrogen from immobilized enzymes, a microreactor was never fabricated. It was decided that time and effort should be

focused on optimizing the immobilization procedure before time and expense were spent on the production of the microfabricated reactor. Despite never fabricating the reactor, several conceptual designs were developed that could eventually be fabricated. One of these designs is shown schematically below (see Figure 9).

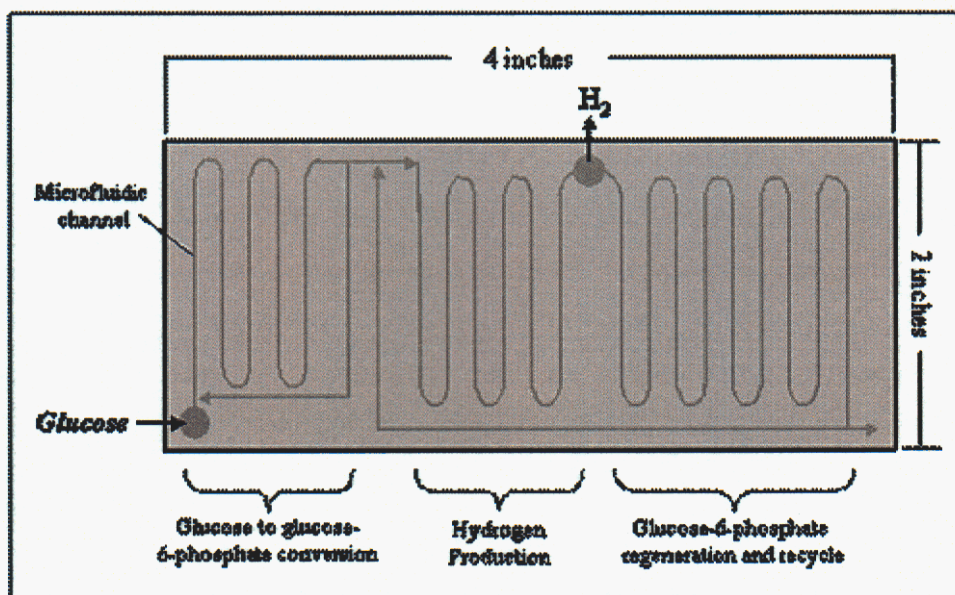


Figure 9: Conceptualized schematic of microreactor for the complete conversion of glucose to H₂.

Conclusions

This work demonstrated that glucose could be enzymatically converted to hydrogen. Unfortunately, this work was unable to advance the work done previously at ORNL; initial attempts to create an efficient hydrogen production system based on immobilized enzymes and capable of operating in a continuous mode (as opposed to batch mode) proved unsuccessful. Despite this lack of success, this project did yield some promising results that could be built upon in the future. There are several major challenges that must be overcome for the microbioreactor envisioned in this work to come to fruition. First, the kinetics of hydrogen production will have to be increased dramatically (by an order of magnitude or more). Achieving this large increase in production rates will require development in multiple areas. This includes using enzyme engineering to increase the activity of the key enzymes in the system. In addition, an immobilization protocol will need to be developed that not only maintains the specific activity of the enzyme, but also maximizes the enzyme loading. Finally, thermodynamically, a higher rate of hydrogen production could be achieved if a higher reaction temperature could be used. The hydrogenase enzyme was isolated from a thermophilic organism and has maximal activity at 80°C. Unfortunately, all of the other enzymes used in the system (both in this work and the work done at ORNL) are from

mesophilic organisms and denature at temperatures above 50°C. Current work at ORNL is looking at isolating PPC enzymes from a thermophilic organism in order to run the hydrogen generating reaction at higher temperature (though this may make the system less applicable to powering a microdevice). Nevertheless, with the merging of the micro- and nanotechnology and biotechnology fields, the concept of mimicking biology to harvest energy from the environment is an area that absolutely merits further investigation.

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